Heterogeneity of pluripotent stem cells shows a basic manner of self-renewing stem cells

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Abstract

Pluripotent stem cells that can indefinitely propagate *in vitro* are derived from groups of pluripotent cells in pre- and post-implantation embryos. Mouse embryonic stem (mES) cells and post-implantation epiblast-derived ES (mEpiS) cells, both pluripotent stem cells, are derived from the inner cell mass (ICM) of blastocyst and the epiblast of post-implantation embryo, respectively. Although these pluripotent stem cells are thought to be a homogenous cell population, recent studies reveal that there is apparent heterogeneity. This article discusses the significance of the heterogeneity observed in pluripotent stem cells, based on current findings including our studies. Of particular interest, our study detected heterogeneous expression of *Stella*, a marker of ICM and primordial germ cells (PGCs), in both mES cells and EpiS cells. Intensive analyses of heterogeneous *Stella* expression illustrate the nature of pluripotent stem cells.

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Heterogeneity of mES cells

mES cells are pluripotent cells originated from the inner cell mass (ICM) of blastocysts. However, unlike the cells *in vivo*, mES cells retain pluripotency and exhibit the capacity for indefinite self-renewal, while the cells *in vivo* undergo differentiation according to a strict developmental program. As long as mES cells are cultured in an appropriate medium, they can undergo self-renewal without compromising pluripotency. For this reason, mES cells are generally regarded as a homogeneous group of cells in the majority of studies. Recent studies reveal, however, that a number of genes, such as *Stella* (or *Dppa3*), *Zfp42*, *Pecam1* and *Nanog*, are heterogeneously expressed in mES cells, apparently showing that mES cells are composed of heterogeneous cell populations. Of these genes heterogeneously expressed in mES cells, we have focused on the nature of heterogeneous expression of *Stella*, a definitive marker of the germ cell lineage. *Stella* expression is first observed in preimplantation embryos, thereafter repressed in the epiblast (Payer et al., 2006; Sato et al., 2002), and subsequently re-expressed only following specification of PGCs (Payer et al., 2006). Based on analysis using Stella: GFP reporter mES cells in which GFP expression is driven by the *Stella* promoter, only 20-30% of the mES cells exhibit Stella-GFP expression (Hayashi et al., 2008). Interestingly, under culture condition supporting self-renewal of mES cells, each sub-population, Stella-GFP-positive and Stella-GFP-negative population, were mutually interchangeable; the purified Stella-GFP-positive (or –negative) population could reconstitute the parental proportion of the heterogeneity. This result is consistent with other studies showing that any of purified subpopulation, for example Nanog-negative mES cell population, can reconstitute the parental proportion of the heterogeneity (Chambers et al., 2007; Furusawa et al., 2004; Toyooka et al., 2008). These clearly demonstrate that mES cells are not a homogeneous cell group but rather exhibit meta-stability on which the cells fluctuate between at least two states.

Gene expression analysis using the subpopulations of Stella-GFP mES cells demonstrated that *Stella*-positive mES cells are closely

related to the ICM, whereas *Stella*-negative cells are more related to the epiblast (Hayashi et al., 2008). Consistent with this observation, expression of other genes showing heterogeneous expression in mES cells, such as *Zfp42*, *Pecam1* and *Nanog* is largely correlated; for example Pecam1-positive cells are enriched in *Nanog* transcripts (Furusawa et al., 2006). Strikingly, all these genes are expressed in the ICM, but not in the epiblast. Combined with the observed meta-stability of mES cells, it is feasible that mES cells in the undifferentiated state fluctuate between an ICM-like and epiblast-like status (Figure 1). It is not the case that epiblast-like mES cells are simply emerging differentiated cells in culture, as isolated epiblast-like cells revert to ICM-like cells
restoring the balance. As described above, mES cells are cells indefinitely

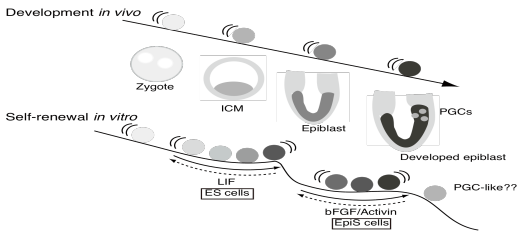

Figure 1. A basic manner of self-renewal of mouse pluripotent stem cells *in vitro* and comparison to their counterparts *in vivo*. After fertilization, cell differentiation irreversibly proceed according to a strict program for development (Development *in vivo*). In contrast, pluripotent stem cells, ES cells and EpiS cells derived from the ICM and the epiblast respectively, can arrest the developmental program under an appropriate condition. To maintain the pluripotent cell population, they fluctuate between subpopulations reflecting the *in vivo* counterpart (Self-renewal *in vitro*).

self-renewing *in vitro*, whereas their origin, the ICM, undergo differentiation according to a defined developmental program. Thus, it is possible that mES cells to some extent mimic the natural differentiation process *in vivo*, leading to the down-regulation of ICM-specific genes and the up-regulation of epiblast-specific genes, and thereafter mES cells revert from the epiblast-like status to the ICM-like status by still unknown mechanism(s). The latter process may in fact be a *bona fide* reprogramming process. Supporting this idea, recent studies demonstrates that mES cells can be derived not only from the ICM of blastocysts at embryonic day (E) 3.5, but also from epiblast cells from later developmental stages, even from E7.5 epiblast, under appropriate culture conditions in the presence of Leukemia Inhibitory Factor (LIF), an important cytokine for mES cell maintenance (Bao et al., 2009). These observations suggest that the appropriate culture conditions evoke a reversion of the process of differentiation. This is quite important to understand the mechanisms underlying the reversion process that illustrates the nature of self-renewal of mES cells.

Considering the intrinsic heterogeneity of mES cells may also be paramount to achieve direct differentiation of mES cells into specific cell lineages, as this heterogeneity may reflect functional differences within the population of mES cells. For instance, our study demonstrated that Stella-positive and Stella-negative cells exhibit distinct differentiation potential. When individual cell populations were cultured under condition promoting trophectoderm differentiation, Cdx2-positive trophectoderm cells emerged from only Stella-negative cells (Hayashi et al., 2008). Although it is generally known that mES cells are not prone to differentiate into trophectoderm cells, our study reveals that such trophectoderm-potent cells are enriched in the Stella-negative population. This may partially explain why it is virtually impossible to induce homogenous differentiation in mES cells, as reactivity to differentiation-inducing factor(s) seems to vary in each sub-population. It is also noteworthy that some, but not all, Stella-negative cells differentiate into trophectoderm cells, indicating that Stella-negative cells can be distinguished into even smaller sub-populations. Making mES cells homogenous is therefore a prerequisite

for directed mES cell differentiation.

Heterogeneity of epiblast stem cells

As described above, mES cells fluctuate between an ICM-like and epiblast-like state under culture condition with LIF. In contrast, recent reports demonstrate that mES cells completely convert into epiblast-like state by cultivation with basic fibroblast growth factor (bFGF) and Activin A, instead of LIF. The converted mES cells indefinitely propagate under these conditions, while keeping pluripotency and features of epiblast cells (Guo et al., 2009). The new pluripotent cells are named epiblast stem (EpiS) cells. EpiS cells are originally derived from E5.5-6.5 epiblast cells and it has been proven, as described above, that EpiS cells can also be derived from mES cells under the appropriate culture conditions (Brons et al., 2007; Guo et al., 2009; Tesar et al., 2007). Under those conditions, the derived EpiS cells no longer contain ICM-like mES cells. EpiS cells express epiblast marker genes and interestingly show epigenetic features similar to those observed in the epiblast. Of those common epigenetic features, it is noteworthy that one of the two X chromosomes is inactivated in female (XX) EpiS cells. In female somatic cells, one of the two X chromosomes is transcriptionally silenced, so that dosage of X-linked gene transcripts is equivalent to that in male cells. However, the germ cell lineage, including pluripotent cells, is the exception. In the case of female mice, both X chromosomes are active in primordial germ cells, the ICM and mES cells. It is suggested that pluripotent cell-specific transcription factors, such as Oct4, Nanog and Sox2, play a role in keeping both X chromosomes active in mES cells and ICM (Navarro et al., 2008). However, despite expression of all the factors in EpiS cells, one of the X chromosomes remains inactive. Thus, it is of particular interest to study how EpiS cells prevent the silent X chromosome from being reactivated.

Given that the fluctuation between sub-populations, reflecting the *in vivo* counterpart, is a common feature of pluripotent stem cells, EpiS cells would be composed of a heterogeneous population that would reflect E5.5-6.5 epiblast. This is the case, as we found at least heterogeneous expression of

Blimp1 in EpiS cells. Blimp1 (or Prdm1) is known as a transcriptional repressor involved in germ cell specification and is the earliest marker for primordial germ cell (PGC) precursors that emerge in the posterior-proximal part of the E6.25 epiblast (Hayashi and Surani, 2009). Using Blimp1-GFP reporter EpiS cell lines, we detected Blimp1-GFP expression in a significant proportion (10 to 50%) of the self-renewing Blimp1-GFP EpiS cells. Correlated with the expression of Blimp1, an early PGC marker gene, transcripts of additional early germ cell marker genes, such as *Prdm14* and *Nanos3*, were selectively enriched in Blimp1-positive subpopulation of the EpiS cells. This finding suggests that a certain subpopulation of EpiS cells undergoes differentiation into *bona fide* PGCs. Indeed, we detected expression of *Stella*, a marker for definitive PGCs, in part of the Blimp1-positive population, though the proportion of Stella-positive cells was rather low (0-2%). Using Stella-GFP reporter EpiS cells, we characterized the population of Stella-positive putative PGCs present in EpiS cells. The putative PGCs capture features of PGCs *in vivo*, as they give rise to EG cells, undergo epigenetic reprogramming and enter meiosis. Interestingly, we also found that in contrast to PGC specification *in vivo* where fate of Blimp1-expressing epiblast cells is basically restricted to PGCs, a part of Blimp1-positive EpiS population was able to give rise to Blimp1-negative EpiS cells and reconstitute the parental proportion. This means not only that similar to mES cells, EpiS cells also fluctuate, while keeping pluripotency (Figure 1); but also that, in aggrement with our previous findings, Blimp1-positive cells are not lineage restricted yet but still need inductive signals to become *bona fide* PGCs (de Sousa Lopes et al., 2007). Although it remains unclear whether other type of subpopulations exist in EpiS cells, it is feasible that EpiS cells fluctuate between several subpopulations corresponding, for example, to anterior and posterior specific lineage progenitors present in the epiblast. This possibility can be evaluated by using anterior or/and posterior epiblast-specific gene reporter EpiS cells.

Human embryonic stem (hES) cells are thought to be close to mouse EpiS cells, rather than to mES cells, as they exhibit similarities with respect to morphology, cytokine requirements and

differentiation potential. Thus, EpiS cells might prove to be a good model to study pluripotency in humans. Consistent with our observation that PGC are spontaneously differentiated from EpiS cells, a recent report has showed that hES cells also generate continuously PGC lineage while keeping pluripotency (Clark et al., 2004; Clark and Reijo Pera., 2006; Bucay et al., 2009). Considering that heterogeneity is commonly observed in pluripotent/multipotent stem cells *in vitro*, it is feasible that hES cells are also composed of heterogeneous cell populations. Using mES and EpiS cells, we propose that each subpopulation has distinct differentiation capacities. Then, in the case of hES cells, it might also be important to control the heterogeneity for directed and homogenous differentiation into a specific cell lineage.

Control of heterogeneity

It has remained unclear how to control heterogeneity. However, it is apparent that the culture condition has a significant impact on the degree of heterogeneity. For example, our study revealed that the proportion of Stella-positive mES cell population increased, when cultured on mouse embryonic fibroblasts (MEFs), suggesting that MEFs push mES cells into a more ICM-like status. On the other hand, culture in chemically defined medium decreased the size of the Stella-positive cell population. Detail analysis suggested that the chemically defined medium placed mES cells at an intermediate position between ICM- and epiblast-like cell populations (in preparation). We are now testing whether these relatively homogenous cell populations exhibit homogenous differentiation into specific cell lineages. As described here, heterogeneity of mES cells show a basic manner of self-renewing pluripotent stem cells *in vivo*. Perhaps, self-renewal, defined as cell division generating two completely identical daughter cells, might not exist in pluripotent stem cells *in vitro*. Further studies are required to fully understand the mechanisms underlying the heterogeneity and its role in pluripotency.

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